BIOCHEMISTRY

A novel TNFR2 agonist antibody expands highly potent regulatory T cells

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Regulatory T cells (T_{reg} cells) restrict immune system activity, such as in response to self-antigens, and are switched on by tumor necrosis factor receptor 2 (TNFR2). Therapeutic activation of TNFR2, thereby expanding T_{reg} cells and suppressing immune activity, may be beneficial to patients with various inflammatory diseases. Here, we characterized a new human TNFR2-directed antibody agonist isolated from mice. We found that the antibody agonist expanded the number of T_{reg} cells within cultures of primary human $CD4^+$ T cells from healthy donors and patients with type 1 diabetes or Sézary syndrome. These T_{reg} cells had increased metabolic gene expression and intracellular itaconate concentrations, characteristics associated with maximally suppressive, anti-inflammatory T_{reg} cells. Furthermore, antibody-expanded T_{reg} cells repressed the activity of primary human $CD8^+$ effector T cells (T_{eff} cells). Epitope mapping suggested that the antibody bound to TNFR2 through a natural cross-linking surface and that T_{reg} cell expansion was independent of the antibody Fc region. In addition, T_{reg} cell expansion was not increased by adding either supplemental TNF ligand or a cross-linking reagent, suggesting that the antibody agonist by itself can elicit maximal activity, a notion that was confirmed by increased secretion of soluble TNFR2. Pending in vivo tests, these features indicate that this TNFR2 antibody agonist has the potential to safely and effectively treat various inflammatory disorders.

INTRODUCTION

Traditional therapies for inflammatory disorders have depended on immunosuppressive medications that globally diminish immune responses. Although these agents are highly effective for many patients, their common and sometimes serious adverse effects, including life-threatening opportunistic infections, combined with the need for high-dose, long-term use have given impetus for the development of more specific and safer immunotherapies.

One relatively recent approach is to enhance the function of regulatory T cells (T_{reg} cells), a small subset of T lymphocytes that express CD4, high-density interleukin-2 (IL-2) receptor (CD25), and inducible levels of intracellular transcription factor forkhead box P3 (Foxp3) (*1*). T_{reg} cells regulate the immune response in health and in diseases such as autoimmunity versus cancer. Enhancement of T_{reg} cell function is considered beneficial for many disease states (*2*–*9*), including autoimmune diseases ranging from type 1 diabetes (T1D), Crohn's disease, ankylosing spondylitis, and arthritis to colitis (*2*–*9*). Greater T_{reg} cell activity may prevent transplant rejection and graft-versus-host disease (*3*, *10*). Last, T_{reg} cell enhancement may play a special role in neurodegenerative disease, including multiple sclerosis, Parkinson's disease, and dementia. The benefit of a T_{reg} cell-targeted therapy to the central nervous system (CNS) may be derived not only from simple removal of the disease process but also by CNS repair and regeneration, as demonstrated also for pancreatic islet regeneration (*8*–*13*).

Tumor necrosis factor receptor 2 (TNFR2) is abundantly expressed on the surface of the most potent subtype of T_{reg} cell (*14*–*15*). TNFR2 is not merely a cell surface marker; rather, it appears to act as a master control switch for potent T_{reg} cell expansion or T_{reg} cell inhibition (*16*). Agonism of the TNFR2 expands T_{reg} cells, whereas antagonism kills them. Agonism culminates in nuclear factor κB (NF-κB)–dependent gene transcription through the DNA binding activity of RelA (p65), RelB, c-Rel, NFkB1 (p50), and NFkB2 (p53) (p52), as well as through the activity of signaling complexes such as TNFR-associated factor (TRAF) and cIAPs (cellular inhibitors of apoptosis proteins), which are associated with cell proliferation (*17*). TNFR2 is also an attractive candidate for controlling the right balance of T_{reg} cells to T effector cells (T_{eff} cells), which are Foxp3-expressing $CD4^+$ or $CD8^+$ T cells. The right balance of T_{reg} cells to T_{eff} cells is necessary for health. Cancer typically is characterized by too many T_{reg} cells and too few T_{eff} cells, whereas autoimmunity is associated with either too few T_{reg} cells or T_{reg} cells of insufficient potency and too many T_{eff} cells (*15*, *18*). Although TNFR2 is underexpressed on T_{reg} cells in diverse autoimmune diseases, the pathogenic cytotoxic T cells (a subpopulation of $CD8^+$ T_{eff} cells) that cause autoimmunity overexpress TNFR2 and are also targeted by agonism that kills these high–TNFR2 density cells (*19*). TNFR2 agonism expands T_{reg} cells but specifically kills pathogenic cytotoxic T cells in diverse human autoimmune diseases (*19*).

TNFR2 targeting carries some additional advantages. Unlike many other T_{reg} cell markers that are also expressed broadly on diverse lymphoid populations, TNFR2 has a far more restricted lymphoid distribution (*20*). A pivotal study in baboons has shown that TNFR2 agonism, in the form of TNF trimeric muteins with only TNFR2 binding, has minimal toxicity (*21*). This stands in contrast to TNFR1, a receptor with ubiquitous bodily expression and severe toxicity with agonism (*22*).

The creation of a novel agonistic antibody to TNFR2 might be, at first glance, straightforward. However, a closer look reveals that progress has been elusive. Little efficacy has been achieved in early results of ongoing cancer trials using agonistic antibodies to members of the TNF superfamily such as OX40, 4-1BB, and GITR (*23*). There is much speculation on why agonistic antibodies are difficult

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to produce (*23*). In some cases, agonistic antibodies under development were produced with full immunoglobulin G1 (IgG1) function and have required Fc receptor activation through dependence on antibody-dependent cellular cytotoxicity (ADCC). In the clinic, TNF superfamily agonism with ADCC antibodies has, in some cases, resulted in liver toxicity. Also, signaling of TNF superfamily receptors requires a strategic clustering of receptors with the right geometry for sufficient signal strength and efficient intracellular signal transduction for intracellular pathway activation (*24*, *25*).

Here, we first examined the differential expression of TNFR2 on T_{reg} cells and T_{eff} cells in cancer and autoimmunity and then, using these cells, characterized in detail a new human-directed agonistic antibody. We found that this antibody induces T_{reg} cell proliferation, specifically of highly potent T_{reg} cells, and efficient downstream signaling, all in a manner that is independent of the Fc region of the antibody, indicating the likelihood of more limited toxicity in vivo than that which to date has hampered the use of human-directed agonists in the clinic.

RESULTS

TNFR2 density on T_{reg} and T_{eff} cells varies by disease

TNFR2 expression on human T cell populations, T_{reg} and T_{eff} cells, isolated from human participants with cancer (a lymphoma subtype known as Sezary syndrome) or autoimmune disease (T1D) demonstrated substantial variation compared to those isolated from healthy controls. According to mean fluorescent intensity (MFI) of the receptor detected with flow cytometry, a significantly higher proportion of T_{reg} cells from cancer participants expressed TNFR2 compared to those from either healthy controls or type 1 diabetics (Fig. 1A). T_{reg} cells from type 1 diabetics expressed a lesser density of TNFR2 compared to those from normal participants or patients with cancer (Fig. 1A), whereas their T_{eff} cells expressed a significantly greater amount (Fig. 1B).

TNFR2 agonist induces proliferation of T_{reg} cells with maximal agonism

As described in Materials and Methods, TNFR2 antibodies were produced in BALB/c mice with peptides and whole extracellular portions of the human TNFR2 protein. Screening for agonistic activity occurred after confirming selective TNFR2 binding with no TNFR1 binding. Agonistic activity was identified by examining fresh human $CD4^+$ T cells for selective $Foxp3^+$ and $CD25^+$ expression using flow methods, typically after a 2- to 4-day incubation period. An increase in cell numbers of T_{reg} cells and an increase in the density (MFI) of both T_{reg} cell markers allowed the selection of the clone with the greatest potential for further functional studies.

When $CD4^+$ T cells from normal blood donors were treated with TNFR2 agonist antibody, dose-dependent proliferation of T_{reg} cells occurred (Fig. 1C and fig. S1A). We previously reported that TNFR2 agonism (using a different antibody) yields expansion of highly potent and homogeneous T_{reg} cells instead of a mixed population of $CD4^+$ T cells in controls and autoimmune participants (*16*, *26*). A mixed population poses safety problems for clinical trials (owing to cytokine secretion, among other factors) and thus faces more regulatory hurdles. When autoimmune $CD4^+$ T cells from type 1 diabetic participants were tested, the TNFR2 agonist antibody was able to proliferate the T_{reg} cells in a dose-responsive manner (Fig. 1D and fig. S1B).

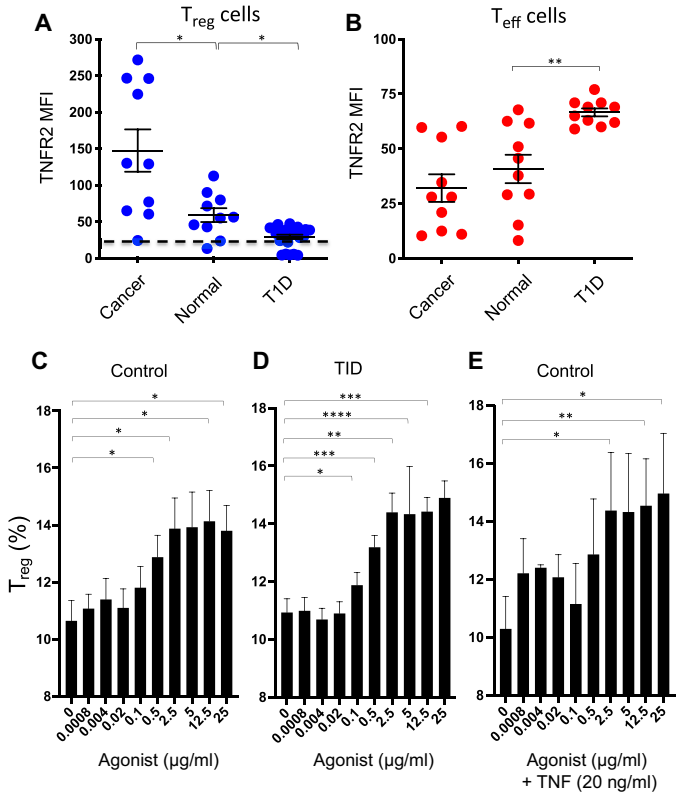

Fig. 1. TNFR2 expression in cancer and autoimmunity and the expansion of T_{reg} cells by the TNFR2 agonist antibody. (**A**) Mean fluorescent intensity (MFI) of TNFR2 abundance on T_{reg} cells isolated from the peripheral blood of cancer (lymphoma, $n = 10$), normal ($n = 10$), and T1D participants ($n = 24$). (**B**) As described in (A) on T_{eff} cells, each $n = 10$. (**C**) Percentage of T_{reg} cells in a culture of freshly isolated peripheral human $CD4^+$ cells from nondiabetic control patients in response to TNFR2 agonist antibody for 48 hours ($n = 13$). Analysis of P values for trend in the linear mixed-effects model (LMEM) up to 2.5 μg/ml, $P = 0.0018$ and greater than 2.5 μg/ml, $P = 0.80$. A representative set of FACS plots is shown in fig. S1A. (**D**) Percentage of T_{reg} cells in a culture of freshly isolated peripheral human $CD4^+$ cells from patients with T1D in response to TNFR2 agonist antibody for 48 hours ($n = 11$). Representative FACS plots are in fig. S1B. (**E**) As described in (C), with added TNF (20 ng/ml; $n = 3$). LMEM analysis, $P = 0.07$. Representative FACS plots are in fig. S1C. In all panels, data are means ± SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$ by Student's t test (unequal variance, two tailed).

When the TNFR2 agonist was combined with TNF at high concentrations (20 ng/ml; Fig. 1E and fig. S1C), expansion of human T_{reg} cell occurred, but it was no greater than that with TNFR2 agonist alone (Fig. 1C and fig. S1A). This demonstrated that supplemental TNF is not obligatory for T_{reg} cell expansion using this form of TNFR2 agonistic antibody. These data suggested that maximal agonism by the TNFR2 antibody may already be present with the antibody without added TNF ligand. This also suggested lower toxicity in human trials. Supplementation with the natural ligand risks high toxicity and has impeded clinical trials with cancer drugs. Although the data are represented as a percentage of cells, for each experiment, total cell number counts were also performed to rule out cell conversion without the total number of cells increasing as well (fig. S2).

One common way to induce T_{reg} cell proliferation is with IL-2 or TNF, although both IL-2 and TNF are difficult treatments in vivo because of their toxicity (*26*). A dose comparison was performed for

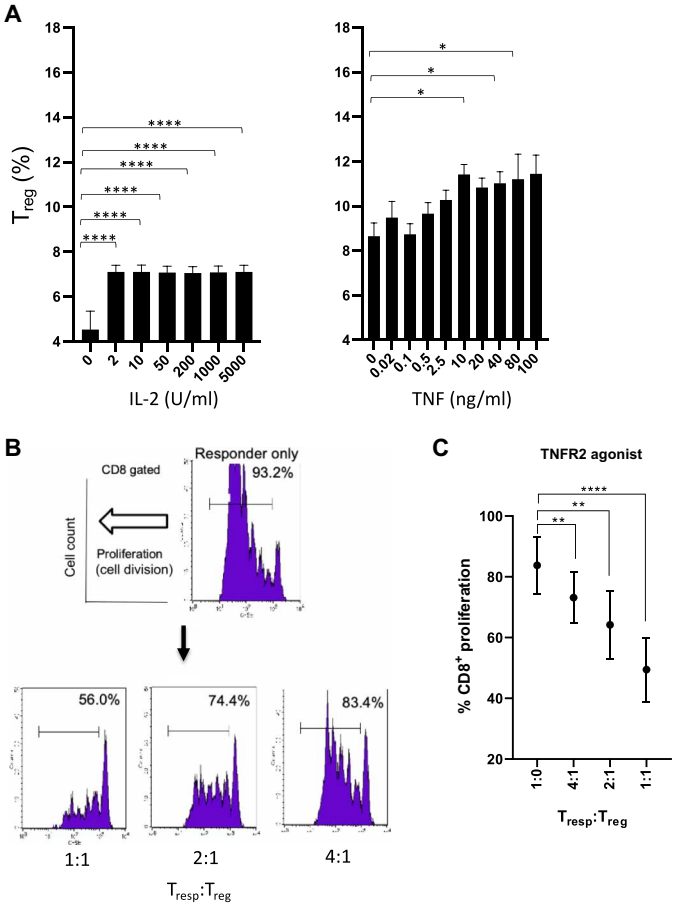

Fig. 2. Comparison of IL-2 and TNF on T_{reg} cell proliferation and the suppressive ability of TNFR2 agonist–expanded T_{reg} cells. (**A**) Expansion of T_{reg} cells (as a percentage) within nondiabetic human $CD4^+$ T cell samples in response to increasing doses of exogenously added human IL-2 (left, $n = 7$ samples) and TNF (right, $n = 8$). (**B**) Functional evaluation, in terms of capacity to suppress $CD8^+/T_{resp}$ cell proliferation, of exogenously TNFR2 agonist antibody-expanded T_{reg} cells added in increasing ratio to $CD8^+$ T cell populations from patients with T1D. Cocultures were stimulated with soluble anti-CD3 (clone Hit3a) and IL-2 (50 U/ml) in 10% FBS RPMI for 4 days. $CD8^+$-gated proliferation in a representative of seven experiments is shown. (**C**) Quantification of the data described in (B), as means ± SEM from seven normal blood donors. $^*P < 0.05$, $^{**}P < 0.01$, and $^{****}P < 0.0001$ by Student's t test (unequal variance, two tailed).

the impact of treatment with IL-2 or TNF alone (Fig. 2A) versus treatment with TNFR2 agonistic antibody (Fig. 1C), which showed the superiority of the latter in expanding human $CD4^+$ T_{reg} cells. It has previously been reported that TNFR2 expansion of T_{reg} cells in culture also generates a more potent T_{reg} cell, not just more T_{reg} cells (*14*). Additional experiments confirmed that the absolute number of T_{reg} cells, not just the percentage of cells, was higher with TNFR2 agonistic antibody (fig. S2). This result rules out the possibility that the percentage increase in T_{reg} cells was solely due to conversion of T_{reg} cells to T_{eff} cells rather than actual T_{reg} cell expansion.

Functional assay reveals that expanded T_{reg} cells suppress $CD8^+$ T cells

To determine the functional capacity of TNFR2 agonist–expanded T_{reg} cells, we tested their ability to suppress autologous $CD8^+$ T cells from patients with T1D. In coculture, we found that the proliferative capacity of the responders—$CD8^+$ T cells (or T_{resp} cells), stained with carboxyfluorescein succinimidyl ester (CFSE)—was inhibited by either a greater proportion of T_{reg} cells compared to responders or by T_{reg} cells expanded in the presence of TNFR2 agonist antibodies. As expected, suppression of responder cell proliferation increased as more TNFR2 agonist–expanded T_{reg} cells were added, as shown in both representative and pooled data from seven donors (Fig. 2, B and C). The TNFR2 agonist antibody was thus effective at generating functional T_{reg} cells in vitro from patients with T1D, known to be inherently defective in generating activated T_{reg} cells (*27*). Similar potency was observed with normal donors (Fig. 1, C and E).

TNFR2 agonist–driven T_{reg} cell expansion does not depend on the Fc region or on a cross-linking agent

Evaluation of the $F(ab')_2$ fragment of TNFR2 agonist antibody demonstrated efficacy at T_{reg} cell proliferation, and therefore, functionality of the antibody is independent of the Fc region (Fig. 3A). Functional activity of the TNFR2 agonist antibody is also independent of receptor cross-linking as demonstrated by coculturing of rodent anti-IgG antibody with TNFR2 agonist, an experiment that did not show greater dose-dependent T_{reg} cell proliferation (Fig. 3B). This finding suggests that the mechanism of action is through an activation pathway that overrides the effect of obligatory cross-linking or Fc binding. It also suggests that treatment with a TNFR2 agonist antibody is likely to be less toxic because it does not require the ADCC mechanism and does not need to be combined with a cross-linking agent. In addition, the lack of reliance on a cross-linking agent suggests that antibody-receptor binding is sufficiently clustered for maximal agonism.

We next studied the TNFR2 antibody agonist for an agonism biomarker, soluble TNFR2 (sTNFR2), in the media. sTNFR2 is a biomarker of maximal agonism developed by the cancer field (*28*–*31*). When there is high-TNFR2 agonism, the membrane receptor is cleaved by TNF-α converting enzyme; this action releases sTNFR2 into the extracellular space and can be used as a biomarker of agonism. We found minimal sTNFR2 in the media with IL-2 alone, some sTNFR2 with TNF, but the most sTNFR2 was seen with TNFR2 agonism (Fig. 3C). This finding is important for translation into the clinic because it suggested that sTNFR2 has potential as a serum-based biomarker of binding efficacy for diseases that do not readily lend themselves to tissue sampling.

TNFR2 agonism induces gene signature indicative of potent T_{reg} cells

Although we have shown that the agonist antibody increases T_{reg} cell numbers and percentages, we do not know from these expansion experiments whether the increases were occurring in the subpopulation of highly potent T_{reg} cells. We therefore selected 33 T_{reg} cell signature genes that characterize potent T_{reg} cells for gene expression after brief and low-dose exposure of $CD4^+$ T cells to TNFR2 agonistic antibody (*32*). This selection of T_{reg} cell-associated genes represents well-described surface proteins and three potent transcription factors that directly control Foxp3 expression. T cell receptor activation resulted in the binding of CREB (cyclic adenosine monophosphate response element–binding protein) to an intronic enhancer element (hereafter referred to as CNS2) in the *FOXP3* gene (*33*). Also, critical is ETS1 binding to the CNS2 region, supporting a

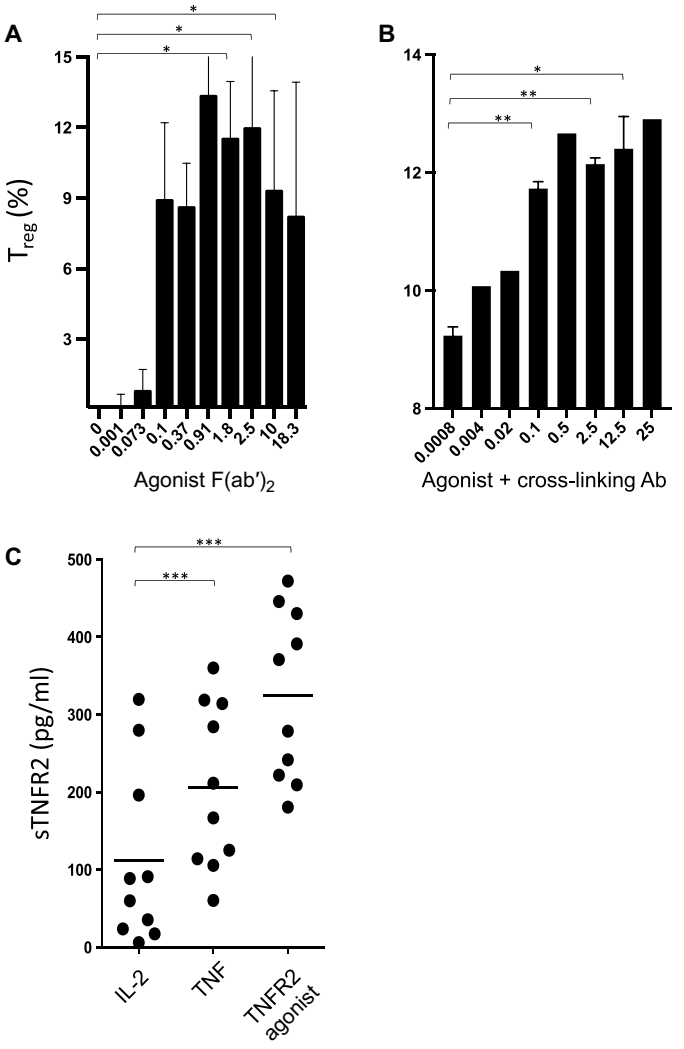

Fig. 3. Expansion of T_{reg} cells in response to F(ab)′ fragments and cross-linking antibody and the impact of TNFR2 agonism on sTNFR2 secretion. (**A**) Treatment of peripheral human $CD4^+$ cells ($n = 5$ samples) with $F(ab')_2$ fragments of TNFR2 agonist antibody with the evaluation of the proliferation of T_{reg} cells relative to treatment with IL-2 alone. (**B**) Incubation of human $CD4^+$ cells ($n = 3$ samples) with cross-linking antibody (ab9165 at 2.5 μg/ml) and examination for augmented T_{reg} cell proliferation capacity of TNFR2 agonist antibody. Similar results were obtained at 24-, 48-, and 72-hour incubation. (**C**) Soluble TNFR2 (sTNFR2), as a measure of TNFR2 shed from brisk agonism, in culture medium from human $CD4^+$ cells ($n = 10$ samples) was evaluated in response to IL-2 alone (200 U/ml), TNF alone (20 ng/ml), or the TNFR2 agonist (2.5 μg/ml). Data are means ± SEM. $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ by one-way analysis of variance (ANOVA) and Tukey's multiple comparison test.

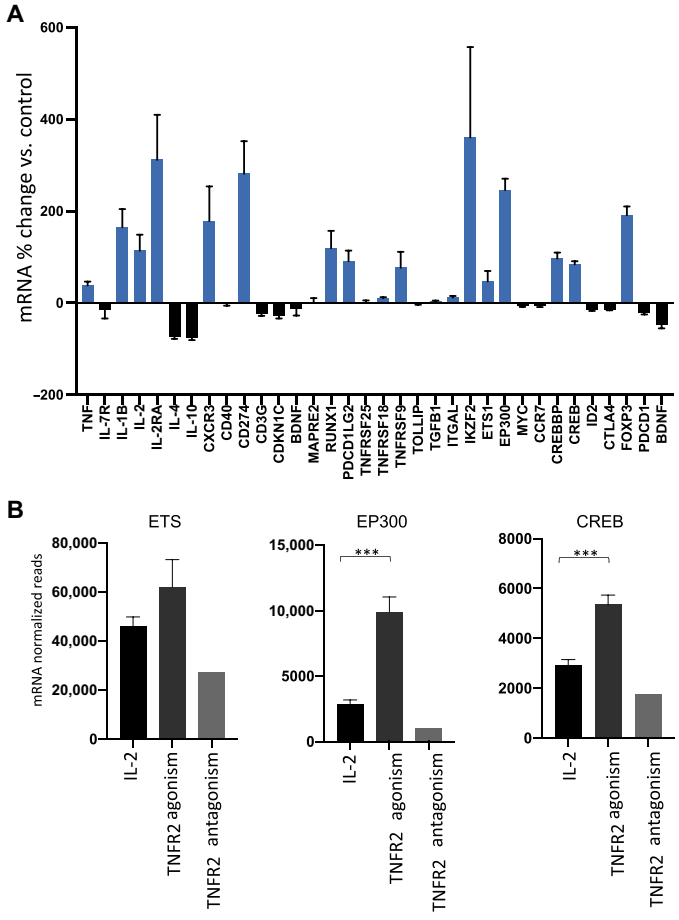

Fig. 4. Induction of T_{reg} cell signature genes after TNFR2 agonism. (**A**) RNA-seq gene expression analysis of key mRNA-encoding proteins known to be important for potent T_{reg} cell function. The graph shows %change in gene expression after treatment with TNFR2 agonist antibody (2.5 μg/ml, 10 hours) as compared to untreated control, performed on purified human fresh $CD4^+$ T cells from nondiabetic controls ($n = 12$). Blue bars represent increases in mRNA after treatment with agonist antibody. (**B**) Effect of the TNFR2 agonist antibody or a TNFR2-antagonist antibody (each 2.5 μg/ml for 10 hours) compared to IL-2 alone on the expression of the FOXP3-inducing genes *ETS1*, *EP300*, and *CREB* in fresh human $CD4^+$ T cells isolated from nondiabetic controls. Data are means ± SEM from $n = 12$ samples. $^{***}P < 0.001$ by Student's *t* test (paired, two tailed).

direct effect of *FOXP3* transcription as well as a role in epigenetic changes required for stable *FOXP3* expression. Histone modification and DNA demethylation are also associated with stable expression of *FOXP3* (*34*, *35*).

Potent T_{reg} $CD4^+$ T cells are known to express panels of proteins related to active and potent suppressor functions. We investigated, using RNA sequencing (RNA-seq) methods, the expression of these T_{reg} cell signature genes in normal $CD4^+$ T cells exposed to the TNFR2 agonist for 10 hours. The panel of known T_{reg} cell signature genes were those that encode nuclear transcription factors that directly stimulate *FOXP3* expression at the important *CNS2* promoter site (ETS, EP300, and CREB) and those that encode common cell surface proteins and secreted cytokines that identify the most potent T_{reg} cells: CTLA4, Helios (IKZF2), Eos (IKZF4), Il2RA (CD25), TNFRSF18 (GITR), TNFR2, PDL2, IL-2, IL-10, IL-4, CD274, MYC, CD3D, CD3G, CDKN1C, MAPRE2, RUNX1, TNFRSF25, TOLLIP, ITGAL, EST1, FOXP3, TNFRSF18, TNF, CTLA4, IRF8, and PDCD1. A 10-hour exposure to TNFR2 agonist induced many T_{reg} cell signature genes (Fig. 4A, blue bars). For statistical significance, an induction of at least 1200 normalized reads was considered significant compared to $CD4^+$ T cells cultured with IL-2 without antibody for the same length of time. Those genes induced rapidly by the agonist included *IL7R*, *IL1B*, *IL2RA*, *CD274* (which encodes PDL1), *MAPRE2*, *RUNX1*, *PDCD1LG2* (encodes PDL2), *TNFRSF9* (encodes 4-1BB), *ITGAL*, *IKZF2* (encodes IKAROS), *ETX1*, *EP300* (which encodes histone acetyltransferase p300, also known as E1A), *CREBBP*, and *FOXP3*.

ETS1, CREB and p300 are all known to interact and directly facilitate *FOXP3* transcription. This pattern of rapid induction of T_{reg} cell signature genes contrasted greatly from the mRNA response of a TNFR2 antagonist (*25*) similarly applied to the same $CD4^+$ isolated cells, also for 10 hours (Fig. 4B). A TNFR2 antagonistic antibody had no impact on the expression of transcripts encoding ETS1, EP300, and CREB and rather decreased that of transcripts encoding ETS1 and EP300 (Fig. 4B). Culturing human $CD4^+$ T cells in media with only IL-2 did not lead to a substantial response. Together, these data are consistent with TNFR2 antagonism suppressing and eventually killing T_{reg} cells (*25*, *36*), as well as inducing highly potent T_{reg} cells. Validating in vivo functional data, however, remains to be obtained.

TNFR2 agonism may induce targeted metabolic changes characteristic of potent T_{reg} cells

Advances have been made in understanding of the metabolism of T_{reg} cells compared to other lymphoid cell populations. Metabolic data combined with T_{reg} cell surface markers allow the most potent T_{reg} cells to be identified and characterized (*16*). To understand this in more detail, in relation to the efficient expansion of human T_{reg} cells with TNFR2 agonism, fresh human T cells were incubated with TNFR2 agonistic antibodies and were studied for changes in metabolism consistent with potent T_{reg} cell induction (10 hours, 2.5 μg/ml of TNFR2 agonist). Differentiated and potent T_{reg} cells display low glycolytic flux and oxidize lipids at higher rates than other T cell subsets (Fig. 5 and fig. S4, A and B) (*36*–*39*).

We then assessed glycolysis and glutaminolysis, at the gene expression level, in response to the TNFR2 agonist antibody. On the basis of down-regulated gene expression in cells from pooled donors, we inferred that various steps of glycolysis were suppressed in $CD4^+$ T cells compared to baseline values, including the associated steps in the pentose phosphate pathway (Fig. 5A and fig. S4A). In contrast, also inferred from gene expression [specifically mRNA sequencing (mRNA-seq) analysis], TNFR2 agonist appeared to up-regulate glutaminolysis in T_{reg} cells (Fig. 5A and fig. S4A). The up-regulation of glutaminolysis would result in augmented amounts of glutamate that can be used in the Krebs (also known as Szent-Györgyi Krebs) cycle; however, this extrapolation from the gene expression-level data remains to be validated at the protein and functional levels.

TNFR2 agonism, through induced adenosine monophosphate kinase and carnitine palmitoyl transferase 1A, augments *FOXP3* expression and may augment the Krebs cycle with fatty acid supply

T_{reg} cells are dependent on mitochondrial metabolism, with the use of oxidized lipid in preference to glucose, whereas other T cells commonly use glucose through glycolysis and do not use fatty acids (*37*–*39*). T_{reg} cells express high levels of carnitine palmitoyl transferase 1a (CPT1a), the rate-limiting enzyme of fatty acid oxidation that allows the entry of acyl groups into the mitochondria. This supports the possibility that T_{reg} cells can use multiple fuel sources. This, too, was observed in TNFR2 agonist–expanded T_{reg} cells; gene expression-level data suggest that TNFR2 agonist–induced T_{reg} cells switched to augmented fatty acids for energy metabolism, given the up-regulation of *CPT1A*, *CPT1B*, and adenosine monophosphate kinase (AMPK) expression (Fig. 5B and fig. S4B). CPT1A also directly induced *Foxp3* expression, another indication of a potent

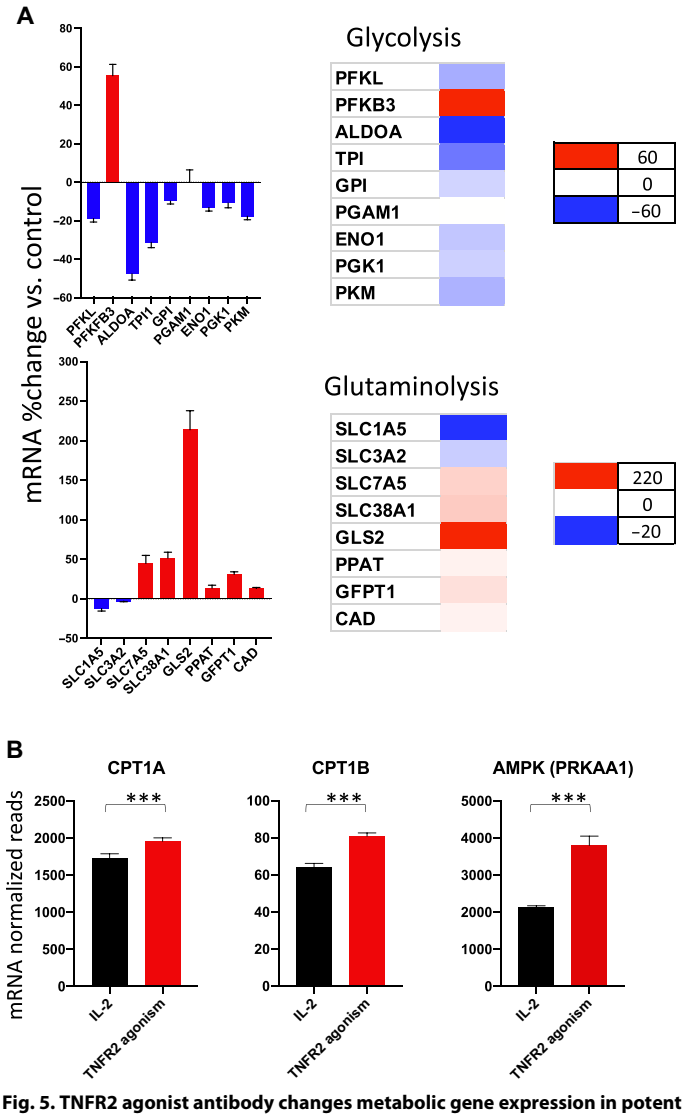

Fig. 5. TNFR2 agonist antibody changes metabolic gene expression in potent T_{reg} cells. (**A**) Human blood derived $CD4^+$ T cells from nondiabetic controls was cultured with and without TNFR2 agonist antibody (2.5 μg/ml) for 10 hours and evaluated for glycolysis- and glutaminolysis-associated mRNA expression. Data are normalized reads of mRNA (%change) compared to untreated control shown as means ± SEM from 12 samples. Heat plots are summaries of the pooled data. (**B**) Expression of fatty acid utilization–associated genes in $CD4^+$ T cells from nondiabetic control patients ($n = 12$) stimulated with TNFR2 antibody agonist (2.5 μg/ml) or IL-2 for 1 hour. Data are means ± SEM. ***$P < 0.001$ by paired Student's t test.

T_{reg} cell signature in response to TNFR2 agonism. These mRNA-seq data are suggestive of increased fatty acid supply and Foxp3 induction, but this remains to be validated at a functional level.

TNFR2 agonism induces early Krebs steps including induction of a possible suppressive metabolite pathway, itaconate

Last, we explored the potential metabolic status of T_{reg} cells upon TNFR2 stimulation in relation to the Krebs cycle. TNFR2 agonism of $CD4^+$ T cells induced gene expression profiles indicative of glutaminolysis and augmented glutamate and fatty acid supply into the Krebs cycle (Fig. 6 and fig. S4C). For example, this is illustrated by

increased expression of *CS*, *ACO1*, and *AC02* mRNA after $CD4^+$ cells were exposed to TNFR2 agonism (Fig. 6), which may correspond to augmented abundance of citrate, oxaloacetate, isocitrate, and acetyl–coenzyme A (fig. S4C). In contrast, gene expression of proteins associated with late steps of the Krebs cycle were not augmented but were rather down-regulated, such as all steps after *IDH2* (Fig. 6 and fig. S4C). Further proof of this shunting to suppressive metabolites from early use of the Krebs cycle was an examination of the mRNA levels of *IL12B* and *KEAP1* that should be down-regulated with this pathway activation. At the mRNA level, both *IL12B* and *KEAP1* were down-regulated within 10 hours of $CD4^+$ TNFR2 agonism exposure (fig. S3), consistent with aconitase pathway usage (fig. S4C). The mechanism proposed remains to be tested further, such as by analyzing T_{reg} cell expansion in the presence of the TNFR2 agonist with AMPK inhibitors or enhancers. Using a low-dose, short-term exposure to the TNFR2 agonist antibody (0.1 to 2.5 μg/ml for 6 hours), we also measured the generation of the suppressive metabolite itaconate. Itaconate is of interest because it has direct immunesuppressive abilities; it has been proposed that the aconitase/itaconate pathway is an immunosuppressive pathway that operates in T_{reg} cell-mediated immunosuppression (*40*). Culture supernatant levels of itaconate increased in a dose-specific response to the TNFR2 agonistic antibody (fig. S3). This was assessed only with a 6-hour, very low-dose exposure to the TNFR2 agonist. Thus, these mRNA data of early utilizations of the Krebs cycle buttressed by direct measurements of the metabolite itaconate support a functional significance of the proposed trends.

Epitope mapping shows that TNFR2 agonist binds to the exterior and cross-links adjacent TNFR2 trimeric complexes

We sought to understand the receptor binding characteristics of potent TNFR2 agonists versus the binding characteristics of many other TNFR2-generated antibodies with no enhanced TNFR2 agonistic functions. During the generation of these antibodies, we gradually observed that immunizations of BALB/c mice with various fragments of the TNFR2 protein led to the more successful generation of agonistic antibody to predominantly, but not exclusively to, only one region of the receptor (Fig. 7). With the use of both enzyme-linked immunosorbent assay (ELISA) peptide mapping of the receptor regions with tightest binding to potent T_{reg} agonistic cell antibody and the use of Pepscan three-dimensional (3D) modeling data, the repeat receptor-binding site of the potent TNFR2 agonists was mostly concentrated within the CRD1 and CRD2 region, as described in Materials and Methods. We propose that, on the cell surface, the ligand-occupied or unoccupied receptor as a trimer is enhanced by single monomeric TNFR2 agonistic antibodies that bind and stabilized adjacent trimeric structures. This is a predictive structure and is consistent with functional data that showed that Fab agonistic antibodies lose their agonistic function but $F(ab')_2$ antibodies retain their function. These stabilized complexes, with enhanced clustering, can then form the cell surface lattice to induce sufficient agonistic stimuli downstream of TNFR2.

TNFR2 agonistic antibody activation likely involves scenario of the pulling together of two adjacent fully trimerized TNFR2 structures. This sort of network then resulted in recruitment of TRAF2 homotrimers inside of the cell (Fig. 7A). Of note is the required separation between TRAF2 trimers to accommodate its zinc-finger and RING domains. This necessitates the separation of the TNFR2 trimers at a sufficient distance and can be bridged by the antibodies to magnify a hexagonal lattice, a structure referred to as a "beehive" from the top down view (Fig. 7A). The orientation of the Fc portion of the antibody may not be exact.

On a functional level, this clustering of surface TNFR2 can magnify NF-κB signaling. This was verified by quantitative polymerase chain reaction (qPCR) and the use of TaqMan Array Human NF-κB pathway mapping plates (Fig. 7B). The panel of TNFR2-regulated genes in this plate included the study of direct target genes of Rel/NFKB family members, namely, *NFKB1*, *NFKB2*, *RELA*, *RELB*, and *REL*, and pathway-associated genes, including those encoding inhibitor of κB (IκB) kinase, IκBs, Toll-like receptor (TLR), TNF, TNFR, and TRAF. Additional genes associated with NF-κB function in apoptosis, immune and inflammation responses, as well as chemokines and cytokines were also included. The representative, pooled data that we present from this analysis show the induction of *BIRC3* and *RELB* gene expression with TNFR2 agonism compared to the same cells incubated with IL-2 alone (Fig. 7B).

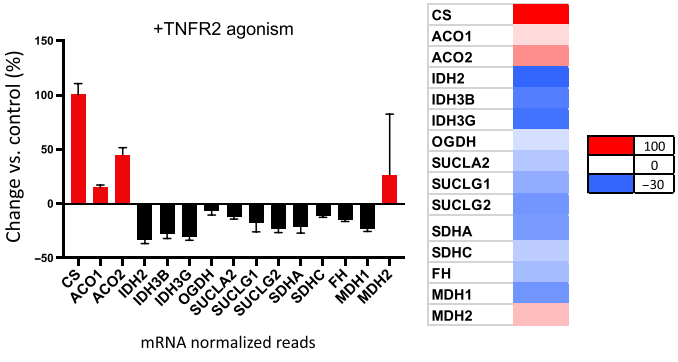

Fig. 6. TNFR2 agonism induces gene expression associated with early steps in the Krebs cycle. Expression of mRNAs associated with Krebs cycle steps in human $CD4^+$ T cells isolated from 12 nondiabetic donors and cultured either with IL-2 ("control") or the TNFR2 agonist antibody (2.5 μg/ml) for 10 hours. Data are shown as means ± SEM (left) %change and as a heatmap averages (right). Genes highlighted in red show up-regulation.

DISCUSSION

In this study, we profiled an agonistic antibody to the TNFR2 and found that it has potential properties for optimized activity. Antibody agonist–induced T_{reg} cell expansion was independent of exterior cross-linking with secondary cross-linking antibodies and was independent of the Fc receptor binding to adjacent cells, meaning an ADCC mechanism. Removal of the Fc region of the antibody demonstrated Fc receptor independence, which is suggestive of lower toxicity for clinical applications. As also shown by previously reported studies on TNFR superfamily antibodies, the agonistic antibody likely creates adjacent dimeric linkages between two fully formed TNFR2 trimeric structures (*24*, *25*). We further propose that this creates stabilizing hexagonal networks for strong intracellular signaling through lattices that appear to look like beehives on the cell surface (*24*, *25*). With only a 10-hour exposure of cultured $CD4^+$ T cells to TNFR2 agonistic antibodies, the induction of well-known T_{reg} cell signature genes was demonstrated. The surface network clustering for maximal agonism was functionally verified by the resulting appearance of sTNFR2 receptors proteins in the media, indicative of

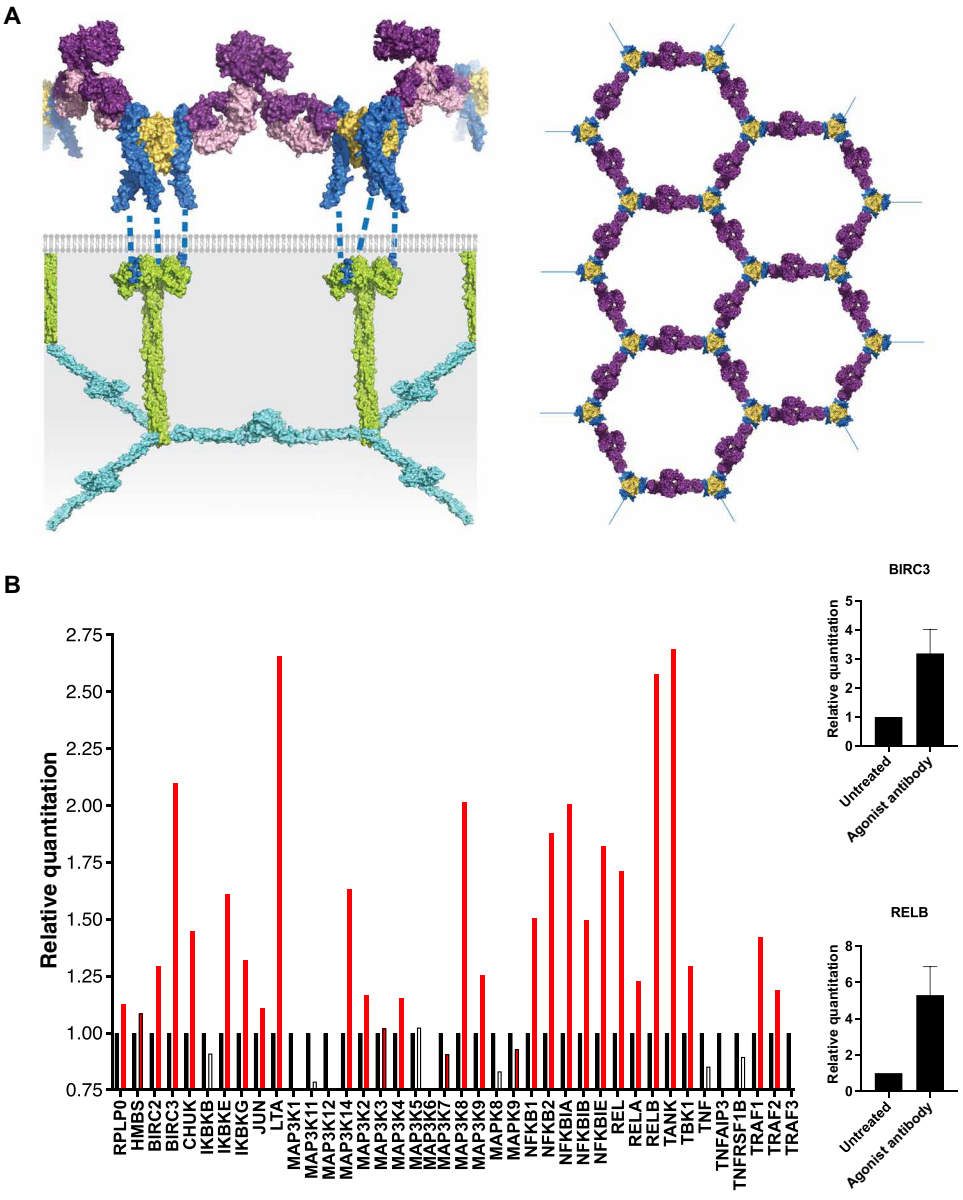

Fig. 7. A structural model of TNFR2 and antibody agonist and analysis of downstream TNFR2 signaling–associated gene expression. (**A**) Schematic representation of the proposed agonist antibody predictive model binding to the TNF-TNFR2 complex. TNF is shown in magenta and TNFR2 in blue in surface representation. Two TNF-TNFR2 complexes are linked and stabilized by an agonist antibody. The antibody heavy chain is shown in purple and the light chain in light purple. Each TNFR2 trimer is bound in the intracellular region to a TRAF2 homotrimer (green-yellow). Two TRAF2 homotrimers are linked via their N-terminal zinc-finger and RING dimerization domains (cyan). The top view of multiple TNF-TNFR2 agonist antibody complexes in the proposed hexagonal beehive lattice arrangement is shown. The TNF-TNFR2 complexes sit at each vertex of the hexagon, and the antibodies (purple) connect two TNF-TNFR2 complexes forming the sides of the hexagon. The structural models were generated with PyMOL (69). (**B**) A NF-κB activation assay assessing downstream TNFR2 signaling–associated gene expression in response to TNFR2 agonism and IL-2 in human donor $CD4^+$ T cells. Red bars denote genes with significant expression over background controls (black bars, cells treated with IL-2 alone), two of which are featured on the right. White bars are TNFR2 genes with background or negative signals. Data are pooled from seven samples.

optimized and tight membrane networks for efficient membrane cleavage of the trimerized receptor. Broad RNA-seq studies of metabolism-related genes in the expanded human T_{reg} cells through TNFR2 agonistic antibody suggested a metabolic switch to a highly potent T_{reg} cells with well-described metabolic activity of induced glutaminolysis, inhibition of glycolysis, augmented fatty acid use, and preferential use of early Krebs cycle steps with augmentation of aconitase signaling. The suppressive itaconate metabolite was induced as well, supporting a functional metabolic effect by the TNFR2 agonist. Future mechanistic studies will be needed to validate these conclusions and should include the reapplication of these methods on sorted and purified T_{reg} cells. It is not known at this time if TNFR2 agonism only expands and increases the potency of existing T_{reg} cells or also recruits $CD4^+$ T cells to become T_{reg} cells as an additional mechanism of mediated suppression.

Early on, it was thought that all agonistic antibodies to the TNF superfamily of receptors required Fc receptor binding (*25*). Our screening of antibodies was independent of Fc receptor binding by performing cellular screening assays on freshly isolated $CD4^+$ T cells, unlike the many Fc receptors on monocytes. In all of these cases, the antibodies presumably stabilize the receptor or receptor-ligand complexes in the membrane with a geometry allowing for efficient TRAF recruitment and receptor activation even when FcRs are absent (*41*–*46*).

Deeper mechanistic insights have emerged as more successful antibody agonists have been made to the TNF superfamily receptors. In the case of CD40 for autoimmunity, the receptor needs to be antagonized; for OX40 in cancer, the receptor needs to be agonized (*44*). Therefore, antibody agonism or antagonism of the TNF receptor superfamily is dependent on linkage of these receptors to death or proliferative pathways. Similar to our TNFR2 agonistic antibodies, CD40-specific agonistic antibodies bind for optimized activity on membrane-distal regions of the CD40 receptor (*45*). Also, some but not all CD40 agonists require Fc receptor function. By contrast, our agonist was largely independent of Fc receptor function. This is important, because Fc receptor requirements of antibodies in vivo may cause heightened liver toxicity or platelet aggregation (*46*). Avoidance of the ADCC dependence is highly desirable to minimize toxicity. Antagonistic antibodies prefer a dimeric form of TNF superfamily members, a form that blocks ligand binding (*24*, *25*). T_{reg} cell proliferation is often accomplished with IL-2, but, as shown, TNFR2 agonism is superior because it relates to total cell numbers and the potency of the expanded

cells. IL-2 alone expansion is weak, and TNF itself can expand the number of T_{reg} cells, but those T_{reg} cells are less potent as we show here and as published in the past (*16*). TNF binds to TNFR1 better than TNFR2; TNFR1 is known in baboons to solely carry the well-known toxicity of TNF (*22*). T_{reg} cell proliferation is also accomplished by the addition of rapamycin; as demonstrated with an earlier TNFR2 agonistic antibody, TNFR2 agonism is superior and addition of rapamycin to a TNFR2 agonism culture is not additive (*16*). A recent review of optimized agonistic antibody properties also highlights the complex monomeric, dimeric binding of antibodies and the need for caution with dosing so that the correct stoichiometry is achieved, typically at low dose, for current cross-linking activities and the avoidance of saturation conditions. It is believed that saturation conditions will culminate in only monomeric interactions that could prevent the proper receptor membrane clustering for intracellular signaling (*23*). On this point, it is important to note that preformed trimeric TNFR2 already in complex with three TNF ligands is already present on the cell surface such that a baseline amount of TNF is sufficient. In this study, we showed that, for optimized TNFR2 agonists, additional TNF ligand is not obligatory, distal binding is preferred, and Fc receptor cross-linking is not necessary.

The literature on antibody development yields some rules about the region of TNF superfamily receptors guiding the function of antibodies that successfully bind. It should be mentioned that these targeted agonists are very different from "blocking" antibodies. Therapeutic blocking antibodies to PD1 and CTLA4 appear to have minimal target receptor region specificity, and most regions of these target proteins with bound antibody have diminished function. This does not seem to be the case for any of the agonistic antibodies to the TNF superfamily receptors that have been reported to date. As future studies progress, it may not only be the receptor sites of TNF superfamily antibodies determining antibody function but agonism may also be determined by the framework structures of the antibodies, with IgG1, IgG2, and IgG4 having different properties influenced by the hinge of the antibody. They feature changes in the antigen-binding arms of antibodies.

The need for TNFR2 agonistic antibodies for diverse human diseases has been strongly documented in animal models and tissue culture models of human disease. Many animal and human in vivo studies in Sjogren's syndrome, rheumatoid arthritis, multiple sclerosis, lupus, ulcerative colitis, T1D, and Crohn's disease show that TNFR2 agonism protects or reverses autoimmunity and may also supplement end organ healing (*4*–*6*, *11*, *13*, *19*, *47*–*54*). It also appears that the "sluggish" or not fully activated T_{reg} cells of human autoimmunity, at least in culture, fully respond to TNF2 agonism, suggesting that the defect might be able to be corrected with this antibody approach (*27*). The literature also shows the value of TNFR2 agonism in reversing graft-versus-host disease and thwarting transplant rejection (*10*, *54*–*59*). The CNS effects of TNFR2 agonism include CNS healing, and this might have applications to Alzheimer's disease, spinal cord injury, and multiple sclerosis (*8*, *9*, *12*, *60*–*66*). The availability of a new TNFR2 agonist that induces highly potent T_{reg} cells with potentially minimal toxicity has wide-ranging potential applications for treatment of inflammatory diseases or diseases with inflammatory components.

A key limitation of this study is that in vivo data are still forthcoming. It is also important to point out that in the clinic, agonistic antibodies to the TNF superfamily have been harder to dose correctly than blocking or antagonistic antibodies. A summary of this literature suggests that lower agonistic dosing, like shown in these in vitro studies, may be more efficacious than high dosing (*23*).

MATERIALS AND METHODS

Research study participants

Human studies that involved blood drawing for autoimmune participants and control participants had full Massachusetts General Hospital (MGH) institutional approval through Massachusetts General Hospital and Partners Health Care (study nos. 2007P001347, 2012P002243, and 2013P002633). Informed consent was obtained from all participants, and the experiments conformed to the principles set out in the World Medical Association Declaration of Helsinki and the Department of Health and Human Services Belmont Report. Whole blood was collected from research study participants in BD Vacutainer EDTA tubes (BD Diagnostics) and processed within 2 hours of phlebotomy. Human blood samples were also received from cancer participants with advanced lymphoma (Sezary syndrome) from the Stanford University Human Studies Committee [IRB (Institutional Review Board) 5538 and IRB 13844].

The donors of all blood samples were adults and spanned in age from 25 to 52 years. Gender information was not part of the informed consent process for all these fresh blood draws. Lymphoma participants were all stage II/IV. At the time of the fresh blood draws, some of these lymphoma participants were receiving therapeutic intervention; however, we found no correlation between TNFR2 MFI and the treatment medication. All donors were provided written informed consent.

Isolation of human $CD4^+$ T cells

For purple top (K_2-EDTA anticoagulant) collection, fresh human whole blood was washed twice with 1× Hanks' balanced salt solution (HBSS) (Invitrogen) plus 2% fetal bovine serum (FBS) (Sigma-Aldrich). $CD4^+$ T cells were isolated using magnetic EasySep Direct Human $CD4^+$ T Cell isolation kits (STEMCELL Technologies, Vancouver, BC, Canada), following the instructions of the manufacturer. Briefly, 1200 μl of Isolation Cocktail and 1200 μl of RapidSpheres were mixed with 24 ml of whole blood in a 50-ml centrifuge tube and incubated for 5 min at room temperature. Then, 24 ml of Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS) was added, and the tube placed into an Easy 50 EasySep magnet. This immobilized the unwanted cells at the side of the tube. After 10 min, the $CD4^+$ T cell enriched suspension was transferred into another tube, and the magnetic separation process was repeated for 5 min with fresh RapidSpheres. The resulting highly enriched $CD4^+$ T cell suspension was transferred into another tube and purified for a third time using the EasySep magnet. The resulting purity of the final $CD4^+$ T cell preparation was >95%. Isolated $CD4^+$ cells were cultured in RPMI GlutaMAX (Life Technologies) with 10% FBS (Sigma-Aldrich) and 1× penicillin-streptomycin (Life Technologies). Cells were seeded in 96-well round-bottom plates at a concentration of 0.2 to 1×10^6 cells per well, treated with various reagents, and incubated for up to 72 hours at 37°C with 5% CO_2. All cell culture experiments included low-level IL-2 (200 U/ml) in the media.

Antibody production, reagents, and flow cytometry

Functional TNFR2 agonistic antibody production and characterizations were conducted over a 7-year time span through screening of hybridomas after BALB/c mice were repeatedly immunized using

fragments of the human (h) TNFR2 protein, specifically a fragment containing amino acids 50 to 122. Mice were also immunized with the whole exterior TNFR2 protein to increase the affinity of the generated clones. All mice were immunized both intravenously and subcutaneously at least five times before the splenic harvest for hybridoma generation. Tail bleed titer checks by ELISA confirmed responding mice. A desired titer of 1:30,000 to 1:100,000 was used for continuing the project with that animal's spleen. Screening of antibody candidates was carried out on fresh human $CD4^+$ T cells in functional assays looking for specific proliferation of T_{reg} cells with methods described within this paper. Supernatant testing was first performed, and then any clones with subcloning that showed remaining activity were then purified. All antibodies of the IgM isotype were discarded; the antibody under study was an IgG1 isotype. Antibody candidates were purified using affinity chromatography followed by PBS solution. The purity of the antibody was >95%. Bacterial endotoxin levels were determined using the Endosafe-PTS system and Endosafe PTS cartridges (Charles River Laboratories), and endotoxin levels were <1 EU/mg before any in vitro antibody testing. Antibody concentration was determined by measuring absorbance at 280 nm and calculated using the standard extinction coefficient 205,500 M^{-1} cm^{-1} (or 1.0 mg/ml = A_{280}). Additional screening also involved confirmed binding to TNFR2, no binding to TNFR1, and binding to TNFR2 amino acids 50 to 122 using ELISAs. Antibody secondary selection was chosen on the basis of candidates that had the most reliable human T_{reg} cell expansion and the least TNFR1 binding (TY101-10) on greater than 25 random human donors. Murine hybridoma immunizations were performed at the Dana Farber Hybridoma Facility or through contract work. Before all experiments, animal care institutional review boards confirmed the safe and humane treatment of mice. Fluorescence-activated cell sorting (FACS) data were processed using FlowJo software (version 10.0.8) and analyzed on Prism (GraphPad Software, La Jolla, CA).

Other reagents and flow cytometry monoclonal antibodies (mAbs) against human TNFR2 and sTNFR2 were produced internally or obtained from external commercial vendors: Antibody MAB2261 (R&D Systems) was used for measuring TNFR2 cell surface expression. The TNFR2 antagonistic antibody used in this paper was produced by our laboratory and is described elsewhere (*25*). Recombinant human TNF and IL-2 were purchased from Sigma-Aldrich. $F(ab')_2$ fragments of mAbs were prepared using Pierce $F(ab')_2$ Preparation Kit (Life Technologies). Cross-linking antibody against rodent IgG (ab9165) was purchased from Abcam. Cells were prepared for flow cytometry using Human T_{reg} cell Flow Kit (BioLegend) according to the manufacturer's instructions. The mAbs or $F(ab')_2$ fragments were conjugated with fluorescent allophycocyanin (APC) using the Lightning-Link APC conjugation kit from Innova Biosciences using the manufacturer's instructions. Fluorescently stained cells were resuspended in 1× HBSS (Invitrogen) and analyzed using a BD FACSCalibur flow cytometer machine (Becton Dickinson). Antibodies used for FACS analysis of T_{reg} cells included Alexa Fluor 488 anti-human FoxP3 (Clone 259D; BioLegend) for intracellular staining of FoxP3 and phycoerythrin anti-human CD25 (Clone BC96; BioLegend) for cell surface staining of CD25. T_{reg} cell populations were assessed by FACS with FL2 (red) versus FL1 (green) and defined as $CD25^{hi}$ and $FoxP3^+$, whereas T_{eff} populations were defined as $CD25^{hi}$ and $FoxP3^-$. For cell suppression assays, responders were stained with CFSE (BioLegend) and CD8 APC (Clone SK1; BD Biosciences) and analyzed by FACS with FL4 (far red) versus FL1 (green).

Measurement of secreted TNFR2

Secreted sTNFR2 was measured from cell culture supernatants using Quantikine ELISA kits (R&D Systems) with some modifications. Briefly, culture supernatants were collected after 24 to 42 hours of incubation of $CD4^+$ cells with IL-2 (200 U/ml) alone or with TNF (20 ng/ml) or TNFR2 mAbs (12.5 μg/ml) and incubated on either the commercial plates or custom plates coated with 2 μg per well of TNFR2-directed antibodies. ELISA was performed according to the manufacturer's instructions. Absorbance at 450 nm was measured using the SpectraMax 190 Absorbance Plate Reader and analyzed with SoftMax Pro 6.3 (Molecular Devices).

Cell proliferation and suppression assays

For peripheral blood leukocytes (PBLs) proliferation experiments, PBLs were stained with 1 μM CFSE. Cells were plated at a density of 2×10^5 cells per well in a 96-well plate precoated with anti-CD3 mAb (5 μg/ml) (OKT3, eBiosciences). Four days later, cells were collected and analyzed by flow cytometry. The proliferation rate was calculated by the percentage of cells undergoing division. For T_{reg} cell suppression assays, autologous peripheral blood mononuclear cells (PBMCs) were used as responder cells. PBMCs were collected at the day of venipuncture by density gradient separation using Ficoll-Hypaque Plus (GE Healthcare, Piscataway, NJ, USA), cryopreserved at −80°C, and thawed at the day before mixed with T_{reg} cells and rested overnight in RPMI 1640 medium supplemented with 1% FBS and IL-2 (10 U/ml). On the following day, responder cells were stained with CFSE (1 μM). Responder cells (5×10^4 cells) and expanded T_{reg} cells were mixed at the ratio of 0:1, 1:1, 2:1, and 4:1 in culture media and stimulated with anti-CD3 mAb (HIT3a, BD Biosciences) and IL-2 (50 U/ml). After 4 days, cells were collected and analyzed by flow cytometry. Suppression index was calculated by the percentage of $CD8^+$ T cells responder cells that underwent division. The suppression index was calculated using the following equation: (T_{reg} cell proliferation without T_{reg} − T_{resp} proliferation with T_{reg}) / T_{resp} proliferation without T_{reg} cell.

Epitope mapping

ELISA was used for linear epitope mapping of TNFR2 agonists on the TNFR2 external membrane protein sequence. Peptides were purchased from GenScript, diluted in coating buffer, and placed on Immulon 4HBX Flat Bottom Microtiter Plates (Thermo Fisher Scientific) at a concentration of 1 mg per well. Primary TNFR2 mAbs (0.1 mg per well) were incubated with substrates. Secondary antibodies against rodent IgGs were used to label the primary mAbs. Absorbance was measured using the SpectraMax 190 Absorbance Plate Reader and analyzed with SoftMax Pro 6.3 (Molecular Devices). 3D peptide mapping was performed using Chemically Linked Peptides on Scaffolds (CLIPS) technology (Pepscan). Briefly, the target protein was converted into a library of up to 10,000 overlapping linear peptides, which were then bound to a solid support and shaped into a matrix of CLIPS constructs. The affinity of the antibody to the various peptide conformations was used to determine the precise discontinuous epitopes. This antibody had a K_D (dissociation constant) of 1.2(2) nM. The CRD1 and CRD2 discontinuous TNFR2 binding sites were QMCCSKCSPGQH, CSSDQVET, and NRICTCRP.

Transcription profiling analysis (RNA-seq)

$CD4^+$ T cells were isolated from peripheral blood using EasySep Direct Human $CD4^+$ T Cell isolation kits (STEMCELL Technologies),

following the instructions of the manufacturer. The cells were then cultured at 37°C in 24-well tissue culture plates at 3×10^6 cells per well in 1 ml of RPMI with GlutaMAX (Life Technologies), FBS [2% (v/v), Sigma-Aldrich] and human recombinant IL-2 (50 U/ml, Sigma-Aldrich) with or without TNFR2 agonist antibodies at a concentration of 2.5 μg/ml. The cells were then harvested and washed twice with PBS (pH 7.4, Ca^{++} and Mg^{++} free). Various incubation times were studied from 6 to 24 hours. Total RNA was then isolated using the RNeasy Plus Mini Kit of Qiagen (Qiagen Sciences) following the instructions of the manufacturer. The RNA concentration was determined on a NanoDrop analyzer (Thermo Fisher Scientific), and the RNA was frozen at −80°C until processed for RNA-seq. RNA-seq was performed at the Center for Cancer Computational Biology of the Dana-Farber Cancer Institute (DFCI, Boston, MA) as well as at the BioMicro Center (BMC) of the Massachusetts Institute of Technology (Cambridge, MA). RNA quality was determined using an Agilent 2100 Bioanalyzer. Poly-A selection was performed using NEBNext Poly(A) mRNA Magnetic Isolation Modules, following the instructions of the manufacturer (New England Biolabs, Ipswich, MA). The resulting mRNA was used for library preparation using NEBNext Ultra Directional RNA Library Prep Kits for Illumina (DFCI) or NEBNext Ultra II Directional RNA Library Prep kits (BMC), following the instructions of the manufacturer (New England Biolabs). The RNA-seq libraries were run on high-sensitivity DNA chips on the Agilent 2100 Bioanalyzer, and the functional concentration of the library was determined through qPCR using KAPA Biosystems Illumina Library Quantification kits. Libraries to be sequenced were pooled at a concentration of 2 nM, then denatured and diluted to a final concentration of 2 pM, and loaded onto the Illumina NextSeq 500. Alignment of the resulting reads against reference genome HG19 was performed using TopHat and analyzed using Cufflinks. The data were normalized for each sequencing laboratory separately using DESeq, part of the R-Bioconductor package. The data were analyzed using the Ingenuity Pathway Analysis software (Qiagen Sciences), the R Statistical Programming language, and Microsoft Excel.

Measurement of itaconate

Culture supernatants were harvested from fresh human $CD4^+$ T cells cultured with TNFR2 (0, 0.1, 0.5, and 2.5 μg/ml) for 6 hours in 96-well plates (100,000 cells per well). Each well in each 96-well plate yielded 50 μl of culture sample. The harvesting of the culture supernatants was from numerous normal blood donors treated in the dose-specific manner ($n = 6$ donors) with TNFR2 agonistic antibody. The culture supernatants were sent to Metabolon (Research Triangle Park, NC) for the analysis of itaconate levels.

Measurement of NF-κB pathway induction

Similar to the $CD4^+$ T cell methods above [fresh human $CD4^+$ T cells cultured with TNFR2 agonistic antibody (2.5 μg/ml) for 6 hours], the Applied Biosystems TaqMan Array Human NF-κB pathway plates were used per manufacturer's instructions to assess the ability of this antibody to activate downstream NF-κB signaling (Thermo Fisher Scientific). The panel of assays in this plate targets genes encoding the proteins of the Rel/NF-κB family signaling pathways (NFKB1, NFKB2, RELA, RELB, and REL) associated with regulatory proteins [including IκBα, IκB kinase, TLR, TNF and TNFRs, and TRAF], and those associated with NF-κB functions in apoptosis, immune and inflammation responses, as well as chemokines and cytokines were also studied.

Binding affinity measurement

The affinity of antibody binding to recombinant human TNFR2 was measured by Biacore Analysis Services (Precision Antibody). The K_D was 1.2(2) nM. Briefly, the antibody was biotinylated at 5:1 stoichiometry with biotinyl-LC-LC-NOSE (Thermo Fisher Scientific) in PBS. Excess biotinylating reagent was removed by centrifugation chromatography, and the biotinylated antibody was captured on 3000 RU of streptavidin surface to a level of 100 RU. Theoretical maximum of signal with TNFR2 with that level of antibody capture was 26 RU, and that signal was reached with a preliminary experiment using 500 nM TNFR2 in the running buffer. Analysis of the kinetics of antigen binding was performed at a flow rate of 60 μl/min with 2-min injections. Antibodies were injected at a concentration of 1 ng/ml to the final capture of 100 RU. The instrument used was Biacore 3000 with the BioCap chip (GE Healthcare). The double reference method was used for analysis. The reference channel contained the identical level of streptavidin.

Statistical analysis

Statistical significance was determined using Student's t test (two-tailed and unequal variance) in Excel (Microsoft) or GraphPad Prism 8 software (GraphPad Software). The significance of dose-response slopes was determined using linear mixed-effects modeling (LMEM) with participant-level random effects. Computations were performed using SAS version 9.4 (SAS Institute Inc.). Confidence levels were set to 0.05. The level of significance is indicated in the figures by asterisks: $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$.

SUPPLEMENTARY MATERIALS

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Fig. S1. Representative FACS data.

Fig. S2. TNFR2 agonism results in T_{reg} cell expansion.

Fig. S3. KEAP1, IL-12B, and itaconate analysis.

Fig. S4. Reference cell metabolism schematics.

Data file S1. RNA-seq data.

View/request a protocol for this paper from *Bio-protocol*.

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A novel TNFR2 agonist antibody expands highly potent regulatory T cells

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An antibody to trigger Treg cells

For patients with autoimmune and other inflammatory diseases, increasing the number and activity of regulatory T cells (T_{reg} cells) may be therapeutically beneficial. Torrey *et al.* identified an antibody that activated the receptor TNFR2, resulting in T_{reg} cell expansion, and repressed the activity of effector T cells (T_{eff} cells) within cultures of patient-derived T cells. Various physical and functional features of the antibody suggested it may be more potent and less toxic than current antibody or ligand-based methods of activating T_{reg} cells. The findings suggest that this antibody has the potential to restore the balance between T_{reg} cells and T_{eff} cells to treat inflammatory disease.

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